AD_____

Award Number: W81XWH-08-1-0232

TITLE:
Modulation of Beta-catenin activity with PKD1 in Prostate Cancer

PRINCIPAL INVESTIGATOR: Meena Jaggi, PhD

CONTRACTING ORGANIZATION: Sanford Research
Sioux Falls, SD 57104

REPORT DATE: April 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
30-04-2011	Final	1 APR 2008 - 31 MAR 2011

4. TITLE AND SUBTITLE
Modulation of Beta-catenin activity with PKD1 in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0232

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jaggi, Meena

meena.jaggi@usd.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sanford Research/USD

Sioux Falls, SD 57104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and Materiel Command

Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
During the 2010-11 funding cycle we have made significant progress on our project. We have published ten papers and fourteen abstracts. In addition to publications, we have obtained collaborative grants from the state (Governor's 2010 initiative), NIH (NCI RO1) and pharmaceutical industries (Merck Pharmaceuticals, Investigator Initiated Grant). We have used transiently transfected C4-2 cell lines to investigate the effect of PKD mutants on the various aspects of cellular functions including cell proliferation, colony formation and motility. The results from these experiments are presented below and provide promising results for further investigation. However, due to the low efficiency of transient transfection of C4-2 cells, we have decided to generate stably transfected C4-2 cell lines using retro-viral mediated transfection system to obtain reliable results. In this study cycle, we have generated retroviral PKD1 mutants in order to identify the domains involved in interaction and modulation of β-catenin activity. The retroviral system is a well established technique that yields much higher efficiency of generation of stable transfect compared to cationic-lipid mediated transfection. We, in collaboration with Dr. Chauhan, have determined the effect of PKD1 on prostate tumor growth in a xenograft mouse model system. We have also determined the effect of Bryostatin-1 on apoptosis and chemo-sensitization. Additionally, we have also discovered a new modulator of PKD1, curcumin, a chemo-dietary agent. Results were presented at the DOD IMPACT and AACR 2011 meetings. Our work related to curcumin pre-treatment strategy of cancer cells induces chemo/radiosensitivity in cancer cells has been published. Based on clinical implications of this novel strategy, this paper was selected for press release.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION

Understanding the basic biology of prostate cancer will provide us with additional critical information necessary to improve existing treatments or to find newer treatments for patients suffering from prostate cancer. While several genetic alterations play specific functions in a cell, some of these genes can have multiple functions. One such gene encodes a protein called β-catenin, which plays a dual role in cancer cells by: a) playing a role in cellular adhesion via another protein called E-cadherin, and b) playing a role in causing cellular division through a set of proteins which cause cells to divide abnormally. In addition to these proteins which β-catenin is already known to associate with, we have discovered a new interaction with a protein called protein kinase D1 (PKD1) in prostate cancer cells. Unraveling this complex interaction of β-catenin with PKD1 in prostate cancer cells may hold the key to understanding the role of a single important protein in causing unregulated cellular division and loss of cellular adhesion – the two fundamental hallmarks of a cancer cell. We have previously made two important discoveries in this field: a) PKD1 levels are lower in advanced prostate cancer which are associated with more aggressive types of cancer, and b) PKD1 interacts with another important protein in cancer cells, β-catenin. These preliminary discoveries in prostate cancer have led us to put forth the current proposal. Our major objective in this proposal is to understand the consequences of binding of PKD1 to β-catenin in tumor development and to study the exact alteration of these proteins in human prostate cancer tissues.

Understanding the details of how cancer causing proteins communicate with each other in a cell will help us intervene in the disease process more effectively. To this end, we propose to study the effect of β-catenin and PKD1 interaction on the cancer cell. We plan to achieve these goals by increasing PKD1 activity in the cell by use of a drug called Bryostatin1, which has already been used in clinical trials in various types of cancers. During 2008-09 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). In brief, we investigated the effect of bryostatin on PKD1 expression, β-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mechanism to improve efficacy of Bryostatin 1 in clinical setting.

During the 20010-11 funding cycle we have made significant progress on our project. We have published 10 papers and fourteen abstracts. In addition to publications, we have obtained collaborative grants from the state (Governor's 2010 initiative), NIH (NCI RO1) and pharmaceutical industries (Merck Pharmaceuticals, Investigator Initiated Grant). In this study cycle, we attempted to isolate stable C4-2 cell lines transfected with PKD1 mutant constructs in order to identify the domains involved in interaction and modulation of β-catenin activity. Due to low efficiency of transfection of these PKD1 mutants, we have moved these constructs in a retroviral system. The retroviral system is a well established technique that yields much higher

efficiency of generation of stable transfect compared to cationic-lipid mediated transfection. Our retroviral constructs have shown higher transfection efficiency in for prostate cancer cells compared to transient tranfection method. We, in collaboration with Dr. Chauhan, have also developed a xenograft mouse model system with the highly metastatic C4-2 cell lines. Our results are very encouraging and we have been able to generate highly vascularized tumors in nude mice. Our initial experiments suggested that PKD1 expression inhibited prostate tumor growth in xenograft mouse model. In addition, we have investigated the effects of PKD1 overexpression on gene transcription using PCR microarray techniques. To determine the correlation of PKD1 expression with prostate cancer progression, we have collected 60 prostate cancer samples and performed PKD1 and β-catenin staining. Our recent studies also suggest that, Bryostatin-1, a PKD1 modulator, efficiently induces chemo-sensitization in prostate cancer cells. In addition, we have also discovered a new method of PKD1 activation by a dietary compound curcumin. Our work related to curcumin pre-treatment strategy of cancer cells induces chemo/radiosensitivity in cancer cells has been published. Based on clinical implications of this novel strategy, this paper was selected for press release. All related studies have been presented at IMPACT and 102 Annual American Association for Cancer Research (AACR) meeting held at Orlando, FL. However, we still need to complete PKD1 and β-catenin staining and molecular interaction studies and that is why we are requesting for 1 year no cost extension of the grant. This no cost extension will allow us to complete our proposed studies.

BODY - Aim 1: Molecular Nature of PKD1 and β-catenin Interaction

Generation of retroviral PKD1 mutants to determine domain of PKD1 that are required for interaction with β-catenin:

We tried to generate stable clones of C4-2 cells with green fluorescent protein (GFP) fused PKD1 constructs in order to study the physiological significance of PKD1 in prostate cancer. We tried to isolate multiple colonies using standard techniques. However, repeated efforts at isolating colonies failed, since the colonies died very soon in isolation. We attempted transfection using multiple cationic reagents like Lipofectamine, Lipofectamine 2000, FuGENE HD, PolyFect and Effectene to raise stable transfection with little success. Table 1 shows comparison between transfection efficiency of Lipofectamine 2000 and FuGENE HD. Among these various transfection reagents, we found FuGENE HD to produce maximal transfection efficiency. However because of low transfection efficiency, we have moved these PKD1 mutant constructs in retroviral system.

Generation of retroviral infected PKD1 mutant expressing C4-2 cells:

The DNA constructs (2µg) of PKD1 mutants tagged with GFP and cloned in pEGFP vector were obtained from our collaborator Dr. Angelika Hausser, University of Stuttgart, Germany. C4-2 prostate cancer cells were infected PKD1 mutant containing retrovirus. The cells were observed for GFP under a fluorescent microscope (Figure 1). After two days of incubation at 37°C/5%CO_2, the cells were trypsinized and used for further experiments. The retroviral constructs have shown higher transfection efficiency in prostate cancer cells compared to conventional transfection methods. The stably transfected C4-2 cell lines were checked for growth, colony formation ability and motility characteristics.

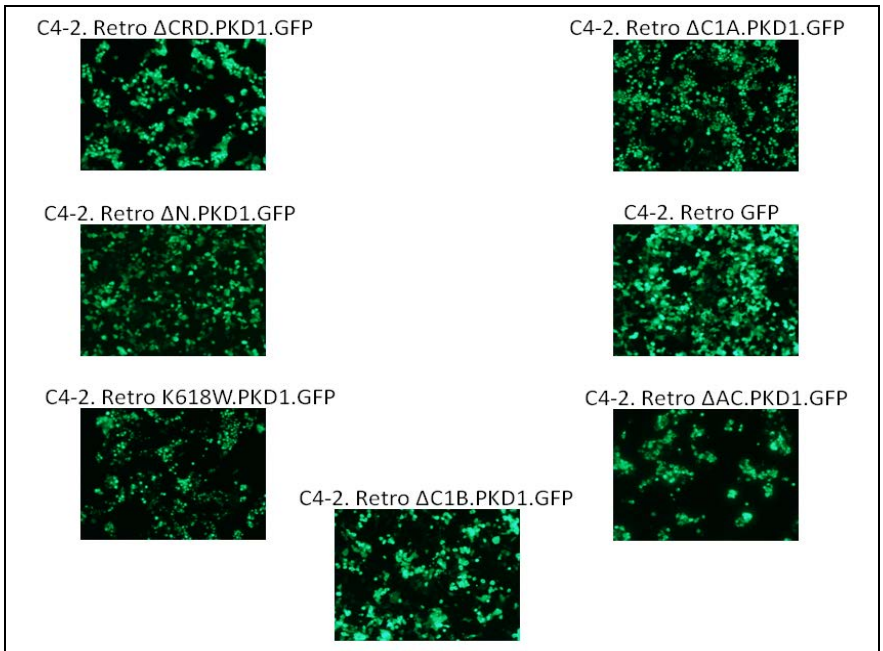

Figure 1. **Retroviral mediated generation of stable C4-2-PKD1 mutant cell lines:** C4-2.PKD1 mutant cell lines were generated using retrovirus mediated transfection. The figure shows fluorescent microscopic images of transfected cells. The deleted domain in each construct is indicated with Δ symbol.

Effect of PKD1mutant constructs on cell motility: The effect of PKD1-GFP mutant constructs on cell motility was assayed using Boyden's chamber. In short, transfected cells were loaded into the chamber in media containing 1% FBS. A chemotactic gradient of 10% FBS was applied to by incubating the chamber in a 6 well plate containing media+10% FBS. Following, incubation of the plates for 24h, the motile cells on the membrane were fixed in methanol for 5min, stained

with crystal violet for 30min, the membrane dried and mounted onto pre-labeled slides. Ten independent images were taken and the motile cells that migrated across the membrane were counted. Our results indicate major difference in the migratory behavior of ΔN, ΔAP and PKD1.KD mutants, indicating the importance of these domains in cell motility (Figure 4). In the next cycle, we plan to generate stable transfects using retroviral system. Once the stable transfects are isolated, the interaction of PKD1-GFP mutant constructs with β-catenin will be analyzed by immunoprecipitation assays, using β-catenin and GFP specific antibodies as described previously. Additionally, the effect of the different constructs on β-catenin transcription will be analyzed.

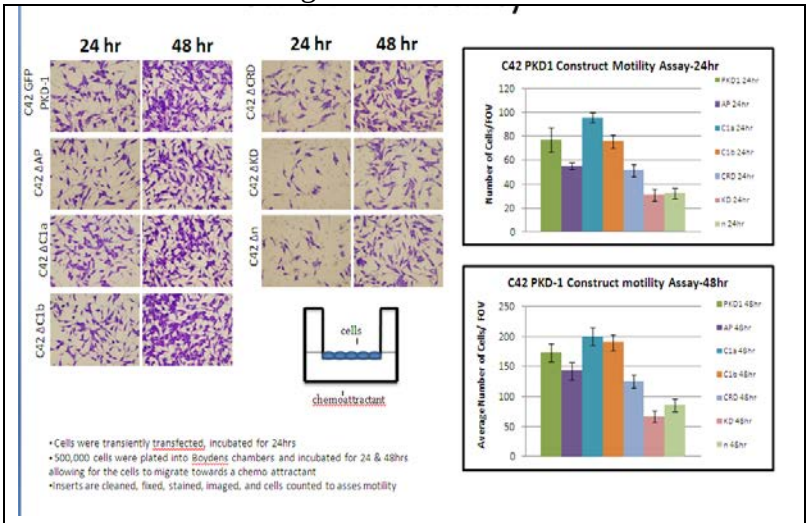

Figure 2. Effect of mutant PKD1 overexpression on cell motility. Boyden's chamber was used to probe the effect of mutant PKD1 expression on C4-2 cell's motility. The cells were plated in 1% FBS onto the chaber and a chemotactic gradient of 10% FBS was was applied for 24h to facilitate cell motility. The cells were fixed, stained and images of ten radom area were captured and the number of cells were counted.

Aim 2: To Demonstrate that Activation of PKD1 by Bryostatin 1 Influences the Cellular Phenotype in Prostate Cancer.

Bryostatin-1: Bryostatin-1, a natural macrocyclic lactone produced by a marine bryozoan, has shown potent anti-cancer properties. It is currently in clinical trials to assess its efficacy as an anti-cancer agent. Bryostatin-1 activates Protein Kinase C (PKC) and Protein Kinase D (PKD) pathways by binding to the phorbol ester binding cystein rich domains and induces effects that are quite different from phorbol ester binding, including biphasic dose response relationship, delayed kinetics and an ability to inhibit phorbol ester mediated response. In this grant we propose to

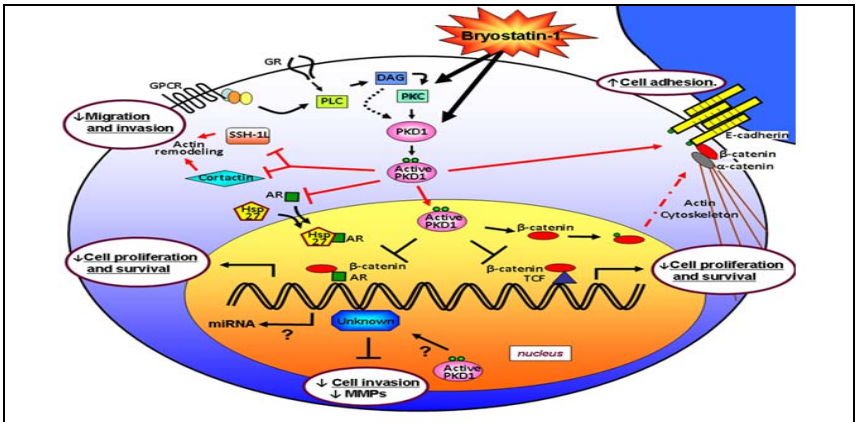

Figure 3: Schematic representation for molecular mechanisms of Bryosatin 1 in prostate cancer cell. Bryostatin 1 (shown in inset) activates PKD1. Active PKD1 phosphorylates β-catenin and thus modulates the transcription and cell-cell adhesion functions of β-catenin. In addition, PKD1 also modulates AR function and transcription activity. PKD1 activation prevents cell migration and invasion through the phosphorylation and inhibition of proteins involved in actin remodeling and cell motility (Cortactin and slingshotphosphatase (SSH1L)). Thus the use of Bryostatin 1 will inhibit tumor growth, metastasis and chemo-sensitize cancer cells to anti-cancer drugs by activating PKD1.

investigate the effect of Bryostatin-1 on prevention and treatment of prostate cancer. We believe that Bryostatin-1 might increase sensitization of the prostate cells to chemotherapeutic agents via altering the β-catenin axis (Figure 3). This approach will improve therapeutic efficacy of chemotherapeutic drugs for prostate cancer treatment.

Time and dose dependent activation of PKD1 by Bryostatin 1

Treatment of the prostate cancer cells stably transfected with PKD1-GFP with increasing concentrations (10-30 nM) of Bryostatin 1 for 3 h demonstrated increased transphosphorylation of ser738 and ser742 and autophosphorylation of ser910 residues of PKD1 (Figure 4 A and B). The activation of PKD1 is phosphorylation-dependent, and serine738 and 742 residues in human PKD1 (corresponding to serine744 and 748 in mouse) have been identified as crucial phosphorylation sites. These serine residues are located in the activation loop of the PKD1 catalytic domain. The C-terminal serine916 residue has been identified as an autophosphorylation site in PKD1 (1). Phosphorylation of these serine residues affects PKD1 activity and plays a role in modulation of PKD1 function *in vivo*. In order to exclude cell line specific effects we also confirmed that Bryostatin 1 activated and is associated with membrane translocation of PKD1 in androgen dependent LNCaP cells (data not shown).

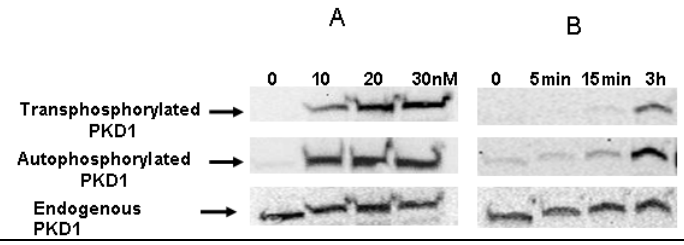

Figure 4: Time dependent phosphorylation of PKD1 in Bryostatin 1 treated cells, **A**, C4-2 cells stably transfected with GFP fused PKD1 (C4-2-PKD1-GFP) were treated with 0, 10, 20 and 30 nM Bryostatin 1. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note optimum phosphorylation at 10 nM. **B**, C4-2 -PKD1-GFP cells were treated with 10 nM Bryostatin 1 for 0-3 h. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note maximum phosphorylation at 3 h.

Bryostatin-1 induces apoptosis in prostate cancer cells: The ability of Bryostatin-1 to induce apoptotic cell death in prostate cancer cells was examined by TUNEL staining followed by flow cytometry. As shown in the figure, longer treatment of C4-2 cells with Bryostatin-1 resulted in higher levels of apoptotic cell death (Figure 5).

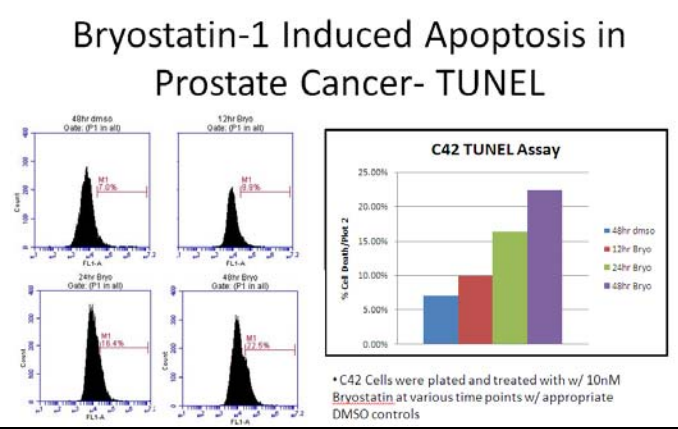

Figure 5: Bryostatin-1 treatment induces apoptotic cell death in prostate cancer cells. C42 cells were treated with 10nM Bryostatin-1 for varying time points. The cells were washed, TUNEL labeled and analyzed by flow cytometry. Representative diagrams of each treatment is depicted on the left. Quantitative analysis of positive TUNEL labeled cells against their appropriate DMSO control is shown as a histogram.

Effect of activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization

Subcellular localization of PKD1, E-cadherin and β-catenin in Bryostatin 1 activated C4-2-PKD1-GFP cells was

analyzed by confocal microscopy. To examine PKD1 specific changes in subcellular localization of E-cadherin and β-catenin, we compared E-cadherin and β-catenin localization in Bryostatin 1 activated C4-2-GFP cells and C4-2-PKD1-GFP cells. In vector transfected C4-2 cells we did not detect any change in E-cadherin or β-catenin localization after Bryostatin 1 activation (Figure 6). Our immunofluorescence study clearly revealed perinuclear and membrane localization of PKD1-GFP upon activation by Bryostatin 1 (Figure 6). The most striking observation was the colocalization of E-cadherin and β-catenin with PKD1-GFP in Bryostatin 1 activated C4-2-PKD1-GFP cells at perinuclear areas in addition to cell membranes (Figure 6, lane2, arrows). After 24 h of Bryostatin 1 treatment, strong membrane staining of E-cadherin/β-catenin and some perinuclear staining was also noticed (Figure 6, lane3). While the C4-2-GFP cells do not over-express PKD1-GFP (Figure 6), they do not show perinuclear localization of E-cadherin/β-catenin. This observation confirms that E-cadherin/β-catenin subcellular distribution is specifically mediated by PKD1 activation and not by other kinases activated by Bryostatin 1.

Bryostatin treatment decreases β-catenin transcriptional activity

We investigated the effect of PKD1 activation on β-catenin mediated transcription activity and proliferation in prostate cancer cells. To investigate the effect of PKD1 on β-catenin mediated transcription activation of TCF, we transfected plasmids containing a wild type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla* luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter (DLR) Assay System. After normalizing the firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. Bryostatin 1 activation in C4-2-PKD1-GFP cells led to a significant reduction (p value=0.019) in β-catenin reporter activity (Figure 7).

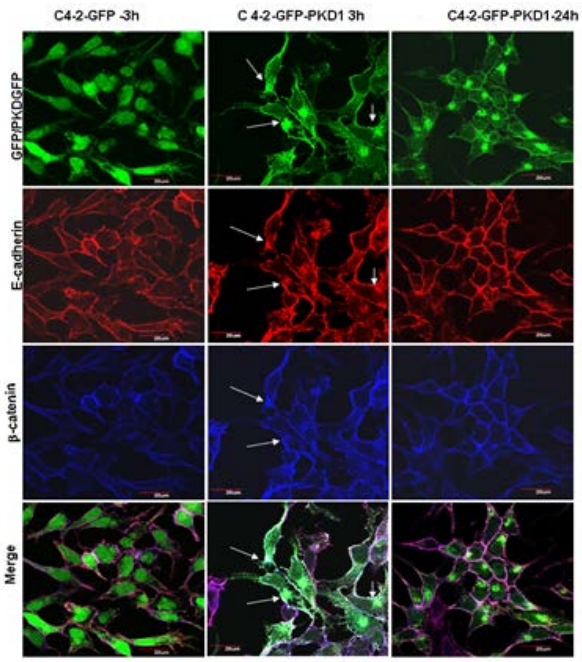

Figure 6: Activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization. Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells were stained for E-cadherin and β-catenin and analyzed by LSM. C4-2-GFP (**3.1**) and C4-2-PKD1-GFP (**3.2** and **3.3**) cells show differences in subcellular localization of E-cadherin (red) and β-catenin (blue). E-cadherin and β-catenin colocalizes with PKD1-GFP at perinuclear areas in addition to cell membranes (**3.2** arrows) in Bryostatin 1 activated C4-2-PKD1-GFP cells (**3.2** and **3.3**) but not in C4-2-GFP cells.

Effect of activation of PKD1 by Bryostatin 1 proliferation

The cell proliferation ability of Bryostatin 1 activated C4-2-PKD1-GFP cells was assayed by CellTiter-Glo. Bryostatin 1 activated C4-2-PKD1-GFP cells showed a 40% decrease in cell proliferation as compared to DMSO treated cells (Figure 7.2). A mixed ANOVA model will be used to compare the cell lines and doses. P-value < 0.05 was considered significant.

Effect of activation of PKD1 by Bryostatin1 on cellular aggregation

PKD1 is also known to be involved with altered cellular aggregation, which is required for a cancer cell to successfully complete the metastatic cascade (2). Because we have demonstrated that PKD1 activation with Bryostatin 1 is involved in trafficking of β-catenin, we sought to determine the effect of Bryostatin 1 activation on cellular aggregation in C4-2 cells over-expressing PKD1. Aggregation assays were performed on C4-2 cells expressing PKD1-GFP as described previously. Our experiments demonstrated increased cellular aggregation in Bryostatin 1 treated C4-2-PKD1-GFP cells compared to vehicle only treated cells (Figure 7.3).

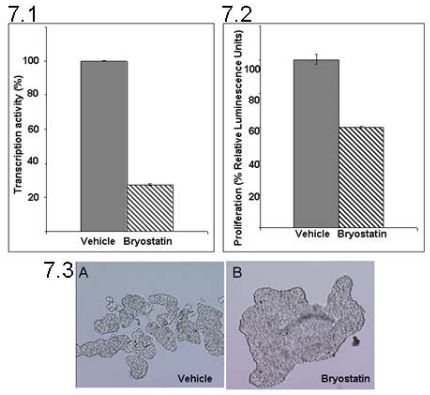

Figure 7: Bryostatin treatment decreases in β-catenin transcription activity, cell proliferation and increases cell-cell adhesion. **7.1)** Effect of Bryostatin treatment on β-catenin transcription activity**.** C4-2-PKD1-GFP cells were transfected with TOP or FOPFlash firefly luciferase reporter constructs and treated with 10 nM Bryostatin 1 or vehicle only. Data are expressed as fold induction normalized to the cotransfected Renilla luciferase-encoding pRL-TK plasmid. Treatment of C4-2-PKD1-GFP cells significantly decreased β-catenin mediated transcription activity as compared to vehicle only treated cells ($p = 0.019$). Error bars indicate standard error. **7.2)** Proliferation assay of Bryostatin 1 treated C4-2-PKD1-GFP cells. Proliferation assay after 48 h of 10 nM Bryostatin 1 treatment (see Materials and Methods) expressed as percent of control cells treated with vehicle only ($p=0.001$). Error bars indicate standard error. **7.3)** Bryostatin treatment increases cellular aggregation. Bryostatin 1 treated C4-2 -PKD1-GFP cells showed increased cellular aggregation (**B**) compared to vehicle only treated cells (**A**).

Effect of PKD1 inhibition on β-catenin subcellular localization

To further demonstrate the specific function of PKD1 in mediating the subcellular redistribution of β-catenin, PKD1 expression was inhibited 90% by using small interfering RNA (siRNA) in C4-2-PKD1-GFP cells activated with Bryostatin 1. After inhibition of PKD1 expression, cells transfected with non-targeted siRNA were activated with Bryostatin 1, stained for β-catenin and trans Golgi network (TGN) specific (p230) antibody and analyzed by confocal microscopy. Non-targeted siRNA transfected and Bryostatin 1 activated cells showed perinuclear localization of PKD1 and β-catenin. Merging of PKD1, β-catenin and p230 images from these cells shows colocalization of these three proteins at the perinuclear region and colocalization of PKD1 and β-catenin at the cell junction. Immunofluorescence images of PKD1 siRNA transfected C4-2-PKD1-GFP cells shows inhibition of PKD1-GFP (Figure 8.E), reduced staining of β-catenin at the membrane and lack of β-catenin localization at TGN (Figure 8 F). Merging of PKD1, β-catenin and p230 images taken at the same confocal level in PKD1 siRNA transfected C4-2-PKD1-GFP cells (Figure 8 H) do not show colocalization of the proteins at the perinuclear region. These results suggest that β-catenin subcellular localization is modulated predominantly by activated PKD1 and not by other kinases (PKC isoforms) activated by Bryostatin 1. Interestingly, down regulation of PKD1 by RNAi decreased β-catenin expression at the plasma membrane (Figure 8 F), which further suggests that PKD1 plays a major role in membrane transport of β-catenin. We have previously published that down regulation of PKD1 in fact increases total cellular β-catenin. This provides further corroborative evidence for role of PKD1 in membrane trafficking of β-catenin because membrane β-catenin is decreased in spite of increased total levels of cellular β-catenin when PKD1 expression is reduced (3). However, the exact mechanism of regulation of β-catenin expression by PKD1 remains to be investigated.

Effect of PKD1 expression on apoptosis:

It has been shown that nuclear β-catenin forms a complex with TCF/LEF transcription factors and that this complex transactivates downstream targets such as *c-myc* and *cyclin D1*. These proteins have been implicated in cell cycle regulation. PKD1 overexpression in C4-2 cells decreases β-catenin/TCF transcription activity. Over expression of PKD1 causes increased cellular aggregation and decreased motility in prostate cancer cells. In order to determine the effect of PKD1 on cell cycle distribution Cell cycle distribution was assessed using BD FACSVantage SE pulse processing plus program for analysis of DNA content. C4-2-GFP vector and C4-2-GFP-PKD1 cells were stained with propidium iodide (PI). Each value represents percentage of cells in the noted cell cycle phase. Experiments were repeated three times and representative histograms are shown. The results show that PKD1overexpression resulted increase of cells in G1 phase and concomitant decrease in cell in G 2 phase, indicate cell cycle arrest in G1 phase.

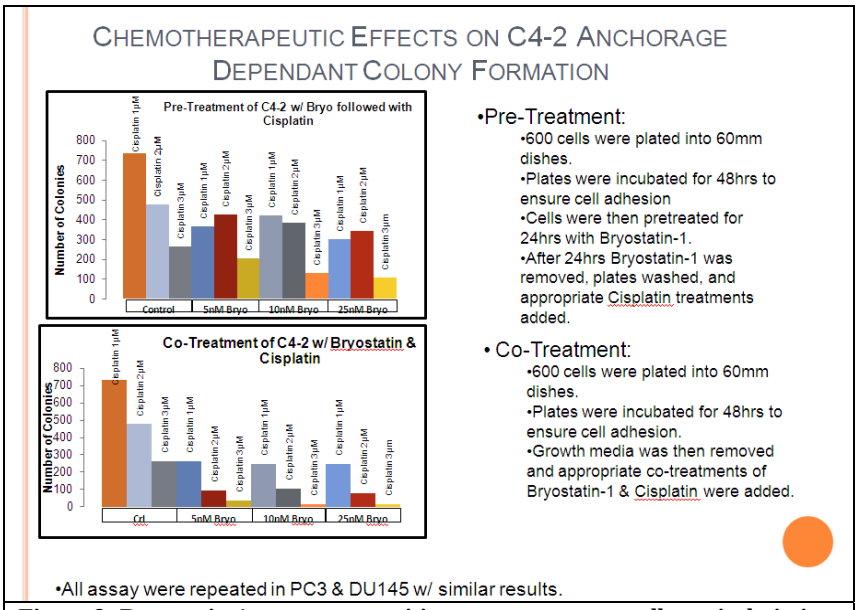

Figure 8: Bryostatin-1 treatment sensitizes prostate cancer cells to cisplatin in a colony formation assay. The effect of pre-treatment and co-treatment are shown in figure.

Bryostatin 1 induces chemo-sensitization in prostate cancer cells: Colony formation assay was used to assess the chemo-sensitizing ability of Bryostatin-1 in prostate cancer cell line model. Briefly, 600 cells were plated onto 60mm dishes. The cells were either pretreated or co-treated with Bryostatin-1 in combination with varying concentrations of cisplatin. Clearly, in this assay, we can see a chemo-sensitizing effect of Bryostatin-1 (Figure 8).

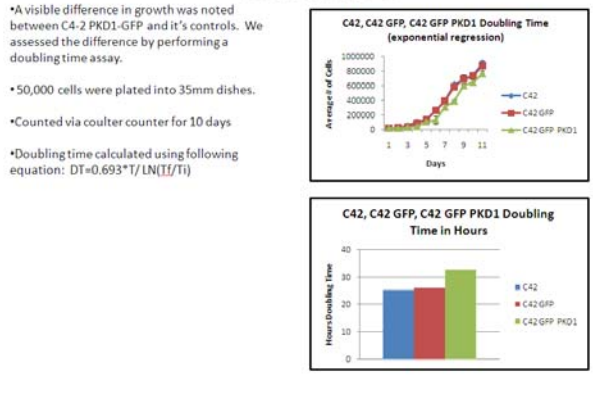

Figure 9: Effect of PKD1 overexpression on growth of prostate cancer cells. GFP or PKD1 overexpressing C4-2 cells were analyzed for cell growth and cell doubling time was calculated. PKD1 expression substantially decreases prostate cancer cell growth.

The inhibitory effect of PKD1 expression on tumorigenicityof prostate cancer cells:
To determine the ability of PKD1 to alter prostate cancer cell growth, cell proliferation and cell doubling time were analyzed. Briefly, $0.5x10^5$ cells were plated in 35 mm dishes and analyzed for cell growth and proliferation by cell counting method (Figure 9). In addition to *in vitro* studies, we performed *in vivo*

tumorigenicity assay using prostate cancer xenograft mouse model. Male athymic Swiss Webster
nude mice, aged 6 to 8 weeks were obtained from Jackson laboratories. Prostate cancer cells with either vector or over-expressing PKD1 were used for tumor development (Figure 10). The tumor growth was monitored bi-weekly for fifty days. At the end of the experiment, all tumors were dissected, measured, weighed and specimen saved at -80^{o}C. PKD1 expression significantly reduced tumor volume (Figure 10). These data suggest a tumor suppressor function of PKD1 in prostate cancer.

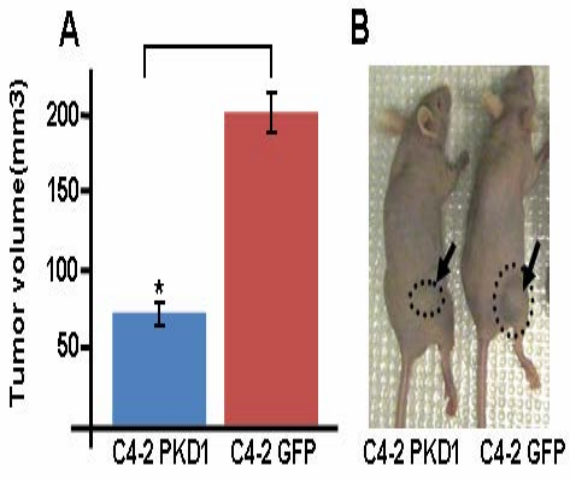

Figure 10: PKD1 expression suppresses prostate tumor growth in xenograft mouse model. To determine if PKD1 suppresses tumorigenesis *in-vivo*, PKD1 overexpressing C4-2-GFP-PKD1 cells and control C4-2-GFP cells ($10x10^{6}$) were injected subcutaneously (s.c) into the flank of athymic nude mice. C4-2 cells expressing PKD1 formed significantly smaller tumors compared to control C4-2-GFP cells.

In addition, we investigated the effect of PKD1 overexpression in a cancer cell line model on gene expression within these cells. Our results show modulation of a number of different genes, five of the highest modulated RNAs are shown (Figure. 11). It is very likely that similar sets of genes are modulated by PKD1 in prostate cancer cells. We would explore this in prostate cancer *in vitro* and *in vivo* model.

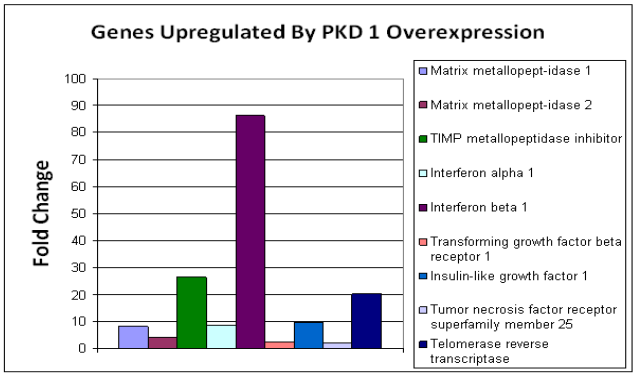

Figure 11. PCR-micro array data: PKD1 overexpression modulates expression of some key tumor associated genes. PCR microarray analysis was done using PKD1.GFP overexpressing cancer cells. Incomparison to GFP vector control cells, we observe dramatic changes in some key genes as represented above.

Aim 3: To evaluate the expression of β-catenin and PKD1 proteins in progressive human prostate cancer:

Our preliminary IHC studies demonstrate that, in addition to downregulation of β-catenin in human prostate cancer compared to benign glands, there is a decreased expression of β-catenin in prostatic intraepithelial neoplasia in a small subset of our study patients and an increased nuclear staining in high Gleason grade prostate cancer. This suggests an involvement of β-catenin and the Wnt signaling pathway in prostate cancer. To determine a correlation between PKD1 and β-catenin expression, we evaluated the expression of these proteins in human

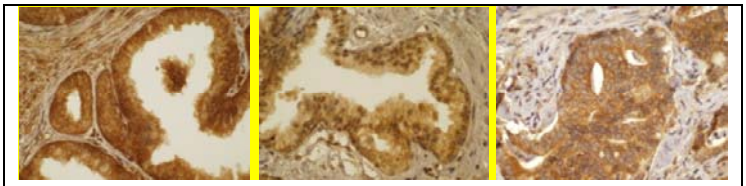

Figure 11. Prostate cancer tissue samples. The samples were probed with PKD1 antibody. Figure shows suppression of PKD1 in Gleason grade 7 (4+3) cancerous areas compared to normal appearing glands. Original magnifications: 400x

prostate cancer tissues utilizing immuno histochemistry (IHC). The cases and controls, matched one-to-one on the basis of patient age and year of biopsy, were included in this study. We have successfully collected 60 prostate cancer patient samples for analysis by IHC. So far, we have stained 60 human prostate cancer tissues for PKD1 and β-catenin protein expression with the Mach4 kit (Figure 11). We are in the process of analyzing the IHC data and correlate with serum PSA, to identify a potential role for these proteins as biomarkers.

Plan for next year:

Generation of Effect of PKD1 mutant constructs* β-catenin *transcription: We will investigate the effect of PKD1 mutants on β-catenin mediated transcriptional activity and proliferation in prostate cancer cells. To investigate the effect of PKD1 mutants on β-catenin mediated transcriptional activation of TCF, we transfected plasmids containing a wild type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla* luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter (DLR) Assay System. After normalizing firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. This study will determine site specific functions of PK
D1 in β-catenin mediated cellular signaling.

Curcumin a new PKD modulator: During this current year, we investigated the effect of many natural compounds that can modulate prostate cancer cells growth and investigated the effects of these on β-catenin transcription activity. Our investigation with curcumin demonstrated that it can attenuates β-catenin transcription activity in prostate cancer cells and can also modulate the expression/activation of PKD1 (Figure 13). These results suggest a novel molecular mechanism of curcumin related suppression of prostate cancer cells growth through modulation of PKD1.

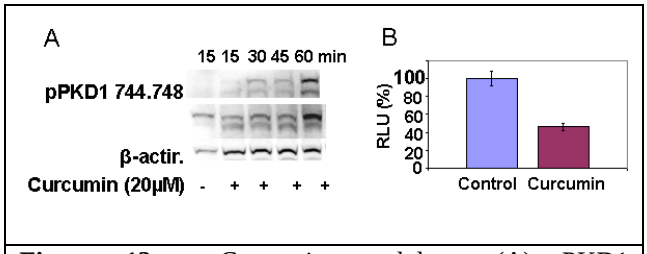

Figure 13: Curcumin modulates (A) PKD1 phosphorylation and (B) β-catenin transcription activity. Treatment of C4-2 cells or C4-2 cells over expressing PKD1.GPP with curcumin showed increase in PKD1 phosphorylation by 60 min. We also detected a ~50% reduction in β-catenin transcription activity (B).

In addition, we investigated the effect of PKD1 overexpression in a cancer cell line model on gene expression within these cells. Our results show modulation of a number of different genes, five of the highest modulated RNAs are shown (Figure. 14). It is very likely that similar sets of genes are modulated by PKD1 in prostate cancer cells. We would explore this in prostate cancer *in vitro* and *in vivo* model.

Key Research Accomplishments: During 2008-10 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). Along with publication few more papers were published from our group. In next funding cycle we are expected to make progress on remaining specific aims and to have couple more publications. In brief, in 2008-10 funding period we have investigated

the effect of Bryostatin 1 on PKD1 expression, β-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Initial activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mchansim to improve efficacy of Bryostatin 1 in clinical setting. We also explored the effect of transient overexpression of various PKD1 mutant constructs on proliferation, colony formation cell motility. Our experiments suggest important role for C1b domain for proliferation and colony formation and the N-terminal region for cell motility. We would like to explore this further using stably transfected cell lines. Also, in collaboration with Dr. Chauhan, we have been able to standardize condition for generation of C4-2 tumor xenograft in nude mice. We will use this model for further studies.

Reportable Outcomes:

- Published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7).
- In 2010-2011, we have published total 10 papers and fourteen abstracts.
- For the first time, we have identified Bryostatin-1 modulates β-catenin signaling through PKD1 and induces chemo-sensitization in prostate cancer cells.
- For the first time, we have shown that curcumin modulated PKD1 activation and subsequent β-catenin transcription activity.

Publication in year 2008-2011:

1. Yallapu MM., **Jaggi M**, and Chauhan SC. Design and engineering of nanogels for cancer treatment. ***Drug Discovery Today*** (In Press) **(*Corresponding Author)**
2. Maher DM., Gupta B.K., Nagata S., **Jaggi M**, and Chauhan SC. MUC13: structure, function and role in cancer pathogenesis. ***Molecular Cancer Research*** (In Press)
3. Maher DM., Bell MC., O'Donnell E., Gupta B.K., **Jaggi M**, and Chauhan SC. Curcumin suppresses Human Papillomavirus oncoproteins, restores expression of p53, pRb and PTPN13 proteins and inhibits benzo[a]pyrene induced upregulation of HPV E7. ***Molecular Carcinogenesis*** 2011;50(1):47-57.
4. Yallapu MM., Othman SF., Curtis ET., Gupta B., **Jaggi M** and Chauhan SC. Multi-functional Magnetic Nanoparticles for Magnetic Resonance Imaging and Cancer Therapy. ***Biomaterials*** 2011 Mar;32(7):1890-905
5. Yallapu MM., **Jaggi M**, and Chauhan SC. Scope of nanotechnology in ovarian cancer therapeutics. ***Journal of Ovarian Research*** 2010, Aug 6;3:19
6. Yallapu MM., Gupta B., **Jaggi M** and Chauhan SC**.** Fabrication of curcumin encapsulated PLGA nanoparticles for improved therapeutic effects in metastatic cancer cells. Journal of Colloid & Interface Science 2010,1;351(1):19-29. **(Cover illustration)**
7. Yallapu MM., **Jaggi M**, and Chauhan SC. β-Cyclodextrin-Curcumin Self-assembly Enhances Curcumin Delivery in Prostate Cancer Cells. ***Colloids and Surfaces B: Biointerfaces,*** 2010 Aug 1;79(1):113-25

8. Yallapu MM., **Jaggi M**, and Chauhan SC. Poly(β-cyclodextrin)-Curcumin Self-assembly : A Novel Approach to Improve Curcumin Delivery and its Therapeutic Efficacy in Prostate Cancer Cells. ***Macromolecular Bioscience*** 2010, 8;10(10):1141-51. **(Cover illustration)**
9. Yallapu MM., Maher DM., Sundram V., **Jaggi M**, and Chauhan SC. Curcumin induces chemo/radio-sensitization in ovarian cancer cells and curcumin nanoparticles inhibit ovarian cancer cell growth. ***Journal of Ovarian Research*** 2010 Apr 29;3:11
10. **Jaggi M**., Du C., Zhang C. and Balaji KC. Protein kinase D1 (PKD1) mediated phosphorylation and subcellular localization of β-catenin. ***Cancer Research*** **2009;69(3)** (1&2 equal contribution)
11. **Jaggi M***., Chauhan SC., Du C. and Balaji KC**.** Bryostatin modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation. **Molecular Cancer Therapeutics** 2008;7(9):2703-12 **(Cover illustration)**
12. Paul M., **Jaggi M**., Viqar S., Chauhan SC., Hassan S., Biswas H., Balaji K.C. Protein kinase D1 (PKD1) influences androgen receptor (AR) function in prostate cancer cells. *Biochemical and Biophysical Research Communications* 2008 Sep 373:618-23.

Abstracts:

1. Nordquist J., Maher DM., Ebeling M., **Jaggi M**. and Chauhan SC. Ormeloxifene treatment inhibits growth of cisplatin-resistant ovarian cancer cells. ***102 AACR Annual Meeting 2011,*** Orlando, FL
2. Gupta BK., Maher DM., Verma R., Ebeling M., Lynch D., Koch M., Watanabe A., Aburatani H., **Jaggi M.** and Chauhan SC. MUC13 expression enhances colon cancer progression. ***102 AACR Annual Meeting 2011,*** Orlando, FL
3. Yallapu MM., Othman SF., Curtis ET., Gupta BK., **Jaggi M**. and Chauhan SC. Multifunctional magnetic nanoparticles for theranostic applications. ***102 AACR Annual Meeting 2011,*** Orlando, FL
4. Hughes JE., Radel S., Sundram V., Jepperson TN., Koch MRD., Chauhan SC. and **Jaggi M**. Protein Kinase D1 expression attenuates colon cancer progression. ***102 AACR Annual Meeting 2011,*** Orlando, FL
5. Yallapu MM., Ebeling M., Maher DM., **Jaggi M.** and Chauhan SC. Targeted curcumin delivery approach for improved prostate cancer therapeutics. **DOD IMPACT Meeting *2011,*** Orlando, FL
6. **Jaggi M**., Sundram V., Radel S. and Chauhan SC. Protein Kinase D1 suppresses B-catenin/T cell factor activity and attenuates prostate cancer growth. ***DOD IMPACT Meeting 2011,*** Orlando, FL
7. Dobberpuhl M., Yallapu MM., Maher DM., Gupta BK., **Jaggi M**. and Chauhan SC**.** Enhancing the efficacy of curcumin for prostate cancer treatment using cellulose nanoparticles. ***8th International Nanomedicine and drug delivery systems-Omaha NanoDDS10***, Oct 3-5, Omaha, NE
8. Yallapu MM, Othman SF., Curtis ET., Gupta BK., **Jaggi M,** and Chauhan SC. Multi-functional Mangetic Nanoparticles for magnetic Resonance Imging and Cancer Therapy. 8th International Nanomedicine and drug delivery systems-Omaha NanoDDS10, Oct 3-5, Hilton Omaha, Omaha, NE
9. Chauhan SC., Ebeling MC., Maher DM., Koch MRD., Friez MH. [4], Watanabe A., Aburatani Hiroyuki., Lio Y., Pandey KK and **Jaggi M**. MUC13 mucin augments pancreatic tumorigenesis. ***101 AACR Annual Meeting 2010,*** Washington DC

10. Maher D., Yallapu MM., Sundram V., Bell MC., **Jaggi M**., Chauhan SC. Curcumin induces chemo/radio-sensitization in ovarian cancer cells and curcumin nanoparticles inhibit ovarian cancer cell growth. ***101 AACR Annual Meeting 2010***, Washington DC.

11. Prakash Preethi., Freiz MH., Bohlmeyer T., Koch MRD., Chauhan SC. and **Jaggi M**. Downregulation of Protein Kinase D1 in hepatocellular carcinoma (HCC): Potential as a diagnostic/prognostic molecular marker. ***101 AACR Annual Meeting 2010***, Washington DC.

12. Yallapu MM., **Jaggi M**. and Chauhan SC. Design of β-cyclodextrin-curcumin self-assembly: A new approach for enhanced curcumin delivery and therapeutic efficacy in prostate cancer cells. ***101 AACR Annual Meeting 2010,*** Washington DC.

13. Sundram V., Chauhan SC. and **Jaggi M**. Curcumin suppresses prostate cancer cells through modulation of β-catenin and protein kinase D1. ***101 AACR Annual Meeting 2010,*** Washington DC.

14. Hughes JE., Chauhan SC., and **Jaggi M.** Protein kinase D1 attenuates tumorigenesis in SW 480 colon cancer cells by modulation β-catenin/T cell factor activity. 3rd ***International Symposium on Translational Cancer Research*** December 18-21, 2009, Bhubaneshwar, Orissa, India

Conclusions:

- Bryostatin-1 treatment modulates PKD1 expression, cell proliferation, and cellular aggregation and alters β-catenin translocation and transcription activity.
- Bryostatin-1 induces chemo-sensitization in prostate cancer cells.
- Initial activation of PKD1 with Bryostatin-1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking.
- Activation of PKD1 by Bryostatin-1 decreases nuclear β-catenin expression and thereby suppresses β-catenin/TCF transcription activity.
- For the first time, we have identified Bryostatin-1 modulates β-catenin signaling through PKD1.
- We have identified curcumin as molecule that can be effectively used for controlling prostate cancer. We have shown for the first time that curcumin modulated PKD1 activation and subsequent β-catenin transcription activity.
- PKD1 overexpression suppresses prostate tumor growth in xenograft mouse model.
- We have collected and stained 60 prostate cancer samples for PKD1 and β-catenin.

Reference:

1. Vertommen, D., Rider, M., Ni, Y., Waelkens, E., Merlevede, W., Vandenheede, J. R., and Van Lint, J. Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis. J Biol Chem, *275:* 19567-19576., 2000.
2. Jaggi, M., Rao, P. S., Smith, D. J., Wheelock, M. J., Johnson, K. R., Hemstreet, G. P., and Balaji, K. C. E-cadherin phosphorylation by protein kinase D1/protein kinase C{mu} is associated with altered cellular aggregation and motility in prostate cancer. Cancer Res, *65:* 483-492, 2005.
3. Mak, P., Jaggi, M., Syed, V., Chauhan, S. C., Hassan, S., Biswas, H., and Balaji, K. C. Protein kinase D1 (PKD1) influences androgen receptor (AR) function in prostate cancer cells. Biochem Biophys Res Commun, *373:* 618-623, 2008.
4. Amundadottir, L. T. and Leder, P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene, *16:* 737-746, 1998.